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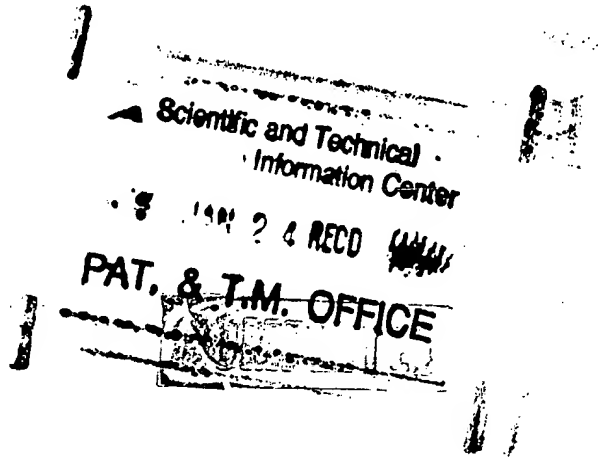

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Ectopic Expression of Bone Sialoprotein in Human Thyroid Cancer

AKEILA BELLAHCÈNE,[1] VALÉRIE ALBERT,[1] LUCA POLLINA,[2] FULVIO BASOLO,[2] LARRY W. FISHER,[3] and VINCENT CASTRONOVO[1]

ABSTRACT

Bone sialoprotein (BSP) is a small, highly posttranslationally modified integrin binding protein found in the mineral compartment of developing bone. The recent discovery that BSP can be detected in a variety of human cancers, particularly those that metastasize preferentially to the skeleton, shed light on potential new biological functions for this protein. The demonstration of a positive association between BSP expression in primary breast tumors and the development of bone metastases suggests that this glycoprotein could play a role in the selective implantation of breast cancer cells in bone. BSP is also expressed in most lung and prostate cancers as well as in multiple myeloma, three other osteotropic malignancies. Because thyroid carcinoma also metastasizes preferentially to the skeleton, we decided to look at the expression of BSP in a collection of 145 thyroid malignant lesions including 24 follicular thyroid carcinomas (FTCs), 55 papillary thyroid carcinomas (PTCs), 19 medullary thyroid carcinomas (MTCs), 23 anaplastic carcinomas (ACs), and 24 poorly differentiated carcinomas (PDCs). BSP expression was evaluated by immunoperoxidase technique using two specific polyclonal antibodies. Most of the thyroid carcinomas (72%) examined expressed high levels of BSP. Expression of BSP was significantly lower in FTCs and MTCs compared with PDCs, which are more aggressive ($p = 0.0009$ and 0.0003, respectively). Our study demonstrates for the first time that ectopic BSP expression is a common feature of thyroid cancer. The prognostic value of BSP detection in thyroid adenocarcinoma and the potential role of BSP in the propension of this type of cancer to metastasize to bone are currently under investigation.

INTRODUCTION

BONE SIALOPROTEIN (BSP) is one of the most prominent noncollagenous proteins of the bone matrix that is implicated in the formation and the remodeling of the mineralized connective tissue matrix (1). BSP was originally thought to be specific to mineralized tissues (2) until the protein was detected in the trophoblasts of the developing placenta (3). Our recent demonstration that BSP—and other noncollagenous proteins, e.g., osteopontin and osteonectin—can be detected in breast cancer suggests new potential functions for this protein during cancer development and progression (4). BSP expression was significantly increased in breast cancer when compared with benign breast lesions (5). Interestingly, a high expression of BSP in primary breast tumors was observed in patients who subsequently developed bone metastases in the course of their disease (6) and was associated with poor survival (7). Ectopic BSP expression was not restricted to breast adenocarcinoma, but was also detected in 74% of human non-small cell lung cancers analyzed (8), in all of the nine human multiple myeloma cell lines examined (9) and in more than 80% of human primary prostate cancers (9A). Moreover, in human ovarian carcinoma, whose metastatic cells rarely disseminate to bone, only a few lesions (21%) were found to express BSP at high levels (8).

Although virtually all cancers can metastasize to bone, it is noteworthy that the majority of bone metastases are due to a few types of cancers, ie, breast, prostate, lung, and multiple myeloma (10). Thyroid cancer also belongs to this restricted group of osteotropic malignancies. The majority of malignant tumors of the thyroid gland are of glandular-epithelial origin and are carcinoma. The principal histological subtypes of thyroid cancers are papillary (PTCs), follicular (FTCs), medullary (MTCs), and anaplastic (ACs) or undifferentiated thyroid carcinoma. While

[1]Metastasis Research Laboratory, University of Liège, Belgium.
[2]Institute of Pathology, University of Pisa, Italy.
[3]Craniofacial and Skeletal Diseases Branch, National Institute of Dental Research, NIH, Bethesda, Maryland.

PTCs, FTCs, and MTCs are considered as good prognosis tumors, ACs are the most aggressive ones and show a near zero 2-year survival in most studies (11). BSP expression has never been investigated in thyroid cancer. This prompted us to examine its expression by immunoperoxidase technique using two specific anti-BSP antibodies in 145 human thyroid carcinoma specimens including both differentiated and undifferentiated thyroid carcinoma subtypes.

MATERIALS AND METHODS

Tissue specimens

A total of 145 human thyroid cancers tissues were obtained from the Institute of Pathology at the University of Pisa, Italy. Specimens were fixed in formalin, embedded in paraffin, and cut into fine sections. The human thyroid tissues examined included 24 FTCs, 55 PTCs, 19 MTCs, 23 ACs, and 24 poorly differentiated carcinoma (PDCs). Adjacent normal thyroid tissue was examined when possible.

Immunohistochemistry

BSP was detected by immunoperoxidase using LF83 and LF100 rabbit polyclonal antibodies raised against synthetic peptides of human bone sialoprotein (residues 277–294 and 129–140, respectively). Both antisera have been previously checked for reactivity by Western blot and shown to react only with BSP (12). Immunoperoxidase was performed using the ABC Vectastain *Elite* kit (Vector Laboratories, Burlingame, CA) according to the supplier's protocol. Briefly, tissue sections were deparaffinized in xylene and hydrated in phosphate buffered saline (PBS, 10 mM sodium phosphate, 0.9% NaCl, pH 7.5). Blocking of the endogenous peroxidase was performed with 0.3% H_2O_2 in methanol and the nonspecific serum-binding sites were blocked with normal goat serum (1:20). LF83 and LF100 antisera at a dilution of 1:1000 and 1:1500 respectively, were applied and incubated for 2 hours at room temperature. Then, the tissue sections were incubated with biotinylated goat anti-rabbit antibody (1:200) followed by exposure to preformed streptavidin-biotinylated horseradish peroxidase complex. Peroxidase was revealed by the 3,3′-diaminobenzidine reaction (SIGMA, Bornem, Belgium). Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. Negative control experiments included omission of the first antibody and pre-incubation of LF100 and LF83 antibodies with an excess of the corresponding or unrelated synthetic peptides prior to their use in the immunoperoxidase assay.

Evaluation of staining

BSP immunoreactivity was evaluated by two independent investigators with no knowledge of the histological results. The degree of staining was evaluated using an arbitrary semiquantitative scale as (0): negative; (1+): focal areas with sparse staining or occasional individual positive cells; (2+): at least one focus with extensive staining or numerous areas with weak to moderate staining; or (3+): extensive staining of more than 50% of the neoplastic cells.

Statistical analysis

The nonparametric Mann-Witney test was used to evaluate the significance of potential differences in BSP expression observed between the thyroid cancer histological groups studied. This analysis was carried using Stat View II™ Version 4.0.2 software (Abacus Concepts, Inc.).

RESULTS

BSP expression was tested in 145 thyroid carcinomas by immunohistochemistry using two different polyclonal antibodies specifically directed against this bone matrix protein (Table 1). The degree and pattern of specific staining was similar in each lesion with both LF83 and LF100 anti-BSP antibodies. The immunoreactivity was mainly cytoplasmic (Fig. 1A) and was abolished by omission of the primary antibodies (data not shown) and by preincubation with a solution containing 100 mol/L excess of the corresponding synthetic peptides as represented in Figure 1B, for LF100 antibody. When present on the tissue section, adjacent normal thyroid tissue was analyzed and exhibited low or no detectable levels of BSP (data not shown). When all subtypes of thyroid lesions were considered, we found that most of the thyroid carcinomas examined (72%) demonstrated a moderate (2+) to high (3+) expression of BSP. Among the differentiated tumors (PTCs, FTCs, and MTCs), PTCs exhibited the highest level of BSP expression (Fig. 1F) when compared with FTCs ($p = 0.0106$) and MTCs ($p = 0.004$). There was no significant statistical difference in the expression of BSP between MTCs (Fig. 1C) and FTCs (Fig. 1D) lesions. All PDCs and ACs, the more aggressive subtypes of thyroid carcinoma, expressed de-

TABLE 1. BSP EXPRESSION IN 145 THYROID TUMORS

	BSP immunoreactivity			
Histotype (n)	*0*	*1+*	*2+*	*3+*
Follicular thyroid carcinoma (24)	2 (8.3)*	7 (29.2)	10 (41.7)	5 (20.8)
Papillary thyroid carcinoma (55)	2 (3.6)	9 (16.4)	15 (27.3)	29 (52.7)
Medullary thyroid carcinoma (19)	1 (5.3)	8 (42)	7 (36.8)	3 (15.9)
Anaplastic thyroid carcinoma (23)	0 (0)	9 (39.1)	5 (21.7)	9 (39.1)
Poorly differentiated thyroid carcinoma (24)	0 (0)	2 (8.3)	6 (25)	16 (66.7)

*Number of cases, percentage in parentheses.

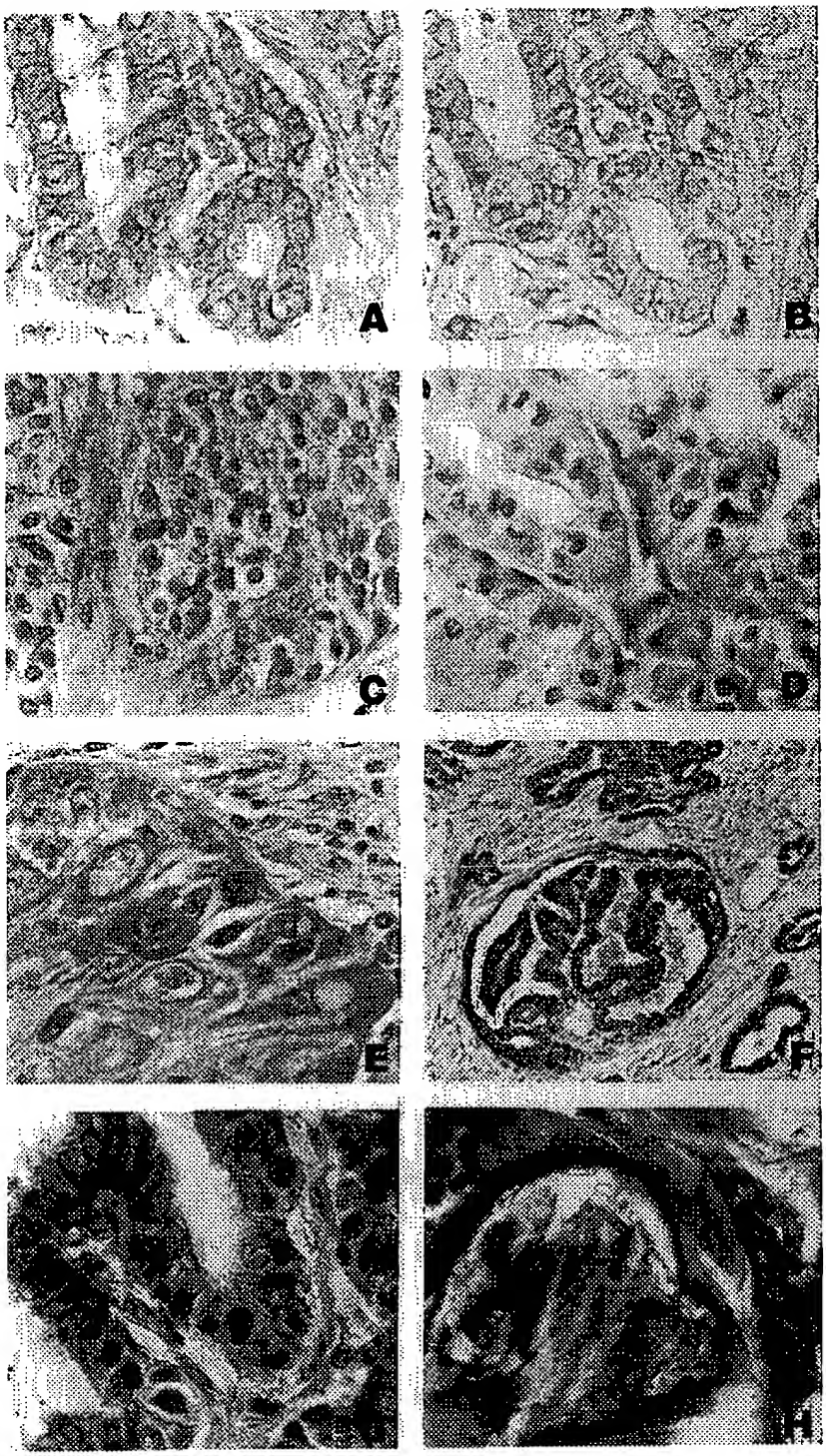

FIG. 1. Detection of bone sialoprotein in human thyroid carcinoma by immunoperoxidase. (A) Representative follicular thyroid carcinoma expressing BSP at a 2+ level. (B) Pre-adsorption of LF100 anti-BSP antibody with its corresponding synthetic peptide results in a significant decrease of immunostaining when applied to the same lesion as shown in A. (C) Medullary thyroid carcinoma and (D) follicular thyroid carcinoma showing a 1+ and 2+ BSP immunostaining, respectively. (E) Anaplastic thyroid carcinoma and (F) papillary thyroid carcinoma usually demonstrated high BSP expression. (G) In some lesions, an intense BSP staining was observed in the Golgi area. (H) Microcalcifications were often adjacent to extracellular zones expressing high levels of BSP. Original magnifications: A, B, C, D, E, G, H: ×400; F: ×100.

tectable levels of BSP. Sixty-one percent of ACs (Fig. 1E) and 91.7% of PDCs expressed 2+ or 3+ level of BSP. PDCs were the thyroid cancer subtype that presented the highest immunoreactivity to BSP antibodies. Indeed, PDCs presented higher amounts of BSP than FTCs ($p = 0.0009$) and MTCs ($p = 0.0003$). Moreover, PDCs appeared to be significantly more immunoreactive to BSP antibodies than ACs ($p = 0.021$). In some of the lesions, a strong BSP staining was clearly visualized in the Golgi area (Fig. 1G). When observed on the sections, microcalcifications were usually surrounded by very positive thyroid cancer cells and some of them were encompassed by a strongly immunoreactive BSP extracellular zone (Fig. 1H).

DISCUSSION

In this study, we demonstrate that BSP is expressed in human thyroid carcinoma. As evaluated by immunohistochemistry, this bone matrix protein is detectable in 72% of thyroid carcinoma studied including PTCs, MTCs, FTCs, PDCs and ACs subtypes. Previously, we demonstrated BSP expression in breast and lung cancers (5,8). BSP expression was also observed in human prostate cancer (9A) and in multiple myeloma (9). All these cancers share the ability to select preferentially the skeleton during metastatic dissemination. Indeed, we have shown that BSP detection in breast primary tumors is significantly associated with the further development of bone metastases (6) and with poor patient survival (7). These data led us to hypothesize that BSP expression in breast cancer cells could be involved in the genesis of bone metastases.

Little is known about the mechanisms responsible for cancer cells osteotropism. However, the interactions of tumor cells with the bone microenvironment are believed to be crucial for the localization and subsequent growth of skeletal metastases (13–15). Several studies describe such interactions of breast cancer cells with bone matrix via integrin receptors (16,17). In analogy to osteoclasts (18), it has been shown that invasive breast cancer cells express the $\alpha v\beta 3$ receptor (19–21). Moreover, a significant increase in this integrin heterodimer has been demonstrated in bone-residing breast cancer metastases (22). Interestingly, MDA-MB-231 breast cancer cells that have been shown to attach to bone via integrin receptors (17) do express BSP (23). In the same way, metastatic follicular thyroid carcinoma cells are capable of binding to bone via integrin attachment molecules (24).

Next to breast and lung cancers, thyroid cancer is the third osteotropic cancer found to express BSP. Tumors of the thyroid gland vary in their biological behavior, degree of differentiation, and prognosis, all of which are closely related to their histological type. Thyroid carcinoma falls into two general types: differentiated and undifferentiated. The majority of all differentiated thyroid carcinomas studied, including PTCs, FTCs, and MTCs was found to express high levels of BSP evaluated as 2+ or 3+ (80%, 62.5%, and 52.7%, respectively). PTCs are the most common form of thyroid cancer. They usually have a favorable prognosis and they rarely generate distant metastases whereas FTCs have a tendency to spread to bone and lungs. Intriguingly, FTCs express significantly less BSP than PTC lesions. If BSP expression is involved in the genesis of bone metastases, it is probably not the only factor that is implicated in this process. Parathyroid hormone-related protein (PTHrP), a powerful stimulator of bone resorption, is expressed in breast cancer (25). Like BSP, the PTHrP molecule is highly expressed in other osteotropic cancers such as thyroid (26) and prostate carcinoma (27). Moreover, PTHrP could contribute to the ability of breast cancer cells to grow as bone metastases. Indeed, the level of PTHrP mRNA expression in breast tumors is significantly higher in patients who later developed bone metastases compared to patients who had no recurrences or metastases and patients who developed soft-tissue metastases (28).

PTCs often produce small calcifications, so-called psammoma bodies (29). In our series, microcalcifications were usually observed in PTCs associated with tumor areas with high levels of BSP. In fact, we have already observed an association between highly immunoreactive mammary malignant epithelial cells and the presence of microcalcifications. In bone, several studies provide evidence that BSP is implicated in the mineralization process (30,31) and this glycoprotein was shown to act as a nucleator of hydroxyapatite *in vitro* (32). We hypothesized, therefore, that the high amounts of BSP expressed by breast and thyroid cancer cells could be responsible for the ectopic formation of calcified deposits in these tumors.

Although there is a discrepancy between BSP expression and the biological behavior of FTC and PTC lesions, we found a consistently high expression of BSP in the more aggressive forms of thyroid cancers (PDCs and ACs), which are the most likely to systemically disseminate. At the opposite of well-differentiated thyroid carcinoma subtypes, none of the undifferentiated and poorly differentiated thyroid lesions analyzed were found to be negative for BSP expression. While both subtypes exhibit high levels of BSP expression, it is noteworthy that PDCs express significantly more BSP than ACs.

In conclusion, we have demonstrated that BSP expression is a common feature of human thyroid carcinoma. The role of BSP in the progression and dissemination of thyroid cancer cells is currently under investigation. Furthermore, the determination of the prognostic value of BSP detection in primary thyroid cancer will require the study of a large cohort of patients with long-term clinical follow-up.

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Address reprint requests to:
Akeila Bellahcène, Ph.D.
Metastasis Research Laboratory
University of Liège
Tour de Pathologie, -1, Bat. B23
Sart Tilman via 4000 Liège
Belgium